REMARKS

Claim 38 is objected to under 37 CFR 1.75(c), as being of improper dependent form for failing to further limit the subject matter of a previous claim. Claim 38 has been cancelled. Accordingly, this rejection is now moot.

Claims 1, 17-19 and 38 stand rejected under 35 USC 103(a) as being unpatentable over Paoletti in view of Ramshaw.

As explained below, both of these references provide very broad descriptions, but fail to provide the specific motivation to combine the references that is required by the relevant Federal Circuit case law.[1] Further, even if one of ordinary skill in the art were to have combined the disclosures of these references there is still no clear suggestion within the references themselves to select the specific combination of elements claimed by applicants. Accordingly, the Examiner's rejection requires one to impermissibly use hindsight reconstruction with knowledge of the invention to pick and choose from the references to arrive at the claimed invention.[2]

Ramshaw was filed in 1987. The specification provides a broad disclosure of co-expression and suggests that HIV antigens and a range of cytokines including interferon-γ may be co-expressed in a range of poxvectors including fowlpox. There is no suggestion in Ramshaw, however, that fowlpox is better than vaccinia or that interferon-γ is better than IL-2. Accordingly, Ramshaw provides no specific motivation to combine Ramshaw with Paoletti or to select the specific combination of elements claimed by applicants.

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[1] As previously explained in the response June 15, 2004, the Examiner's alleged motivation is so general in the context of the relevant art as to constitute no more than the reference to a general level of skill in the art found deficient in *In re Lee*, 277 F.3d 1338, 1343, 61 USPQ2d 1430, 1433 (Fed. Cir. 2002). Under *Lee*, the Examiner must present specific evidence of motivation, not the generalized allegation of motivation relied on in the pending Action.

[2] *See In re Fine*, 837 F.2d 1071, 1075, 5 USPQ2d 1596, 1600 (1988), "[o]ne cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention."

Four years after the filing of Ramshaw, Paoletti was filed. Paoletti discloses three attenuated poxviral vectors comprising various antigens including HIV antigens such as Gag and/or Pol. NYVAC is a vaccinia virus and thus replicates to some extent in mammalian cells. ALVAC and TROVAC are avipox viruses and essentially do not replicate in mammalian cells. ALVAC is a canarypox vector and TROVAC is a fowlpox vector. The Examiner states (page 2, bottom paragraph) that Paoletti "provide[s] fowlpox viral vectors e.g. TROVAC encoding lentiviral e.g. HIV, SIV gene products e.g. Gag, Pol, Env that are suitable for inducing viral specific immune response see col. 5, lines 19 to 35; col. 7, lines 1 to 20; cols. 153 to 154". The Examiner appears to consider that this represents a specific motivation to employ a fowlpox vector encoding HIV Gag/Pol that is suitable for inducing a viral specific immune response. To arrive at this conclusion, the Examiner appears to be importing knowledge obtained from reading applicants' disclosure into his interpretation of Paoletti.

Specifically, the Statement of the Invention, col. 5, lines 19 to 35, of Paoletti states that vaccinia or avipox poxviruses such as fowlpox or canarypox may be used to express gene products of HIV or SIV and may be used as a vaccine for inducing an immunological response. However, in terms of a specific disclosure of the claimed fowlpox vectors, the specification only describes TROVAC comprising HIV genes encoding Nef (Example 33) or Env (Example 3). Murine immunological data are presented in relation to NYVAC and ALVAC vectors, but the specification provides no immunology or treatment data in relation to TROVAC or other fowlpox vectors.

The Examiner refers to Example 3 (Action page 4, line 10) as disclosing the suitability of TROVAC-Env in inducing viral specific immune response. However, the paragraph at the top of column 30 of Paoletti fails to establish that any TROVAC vector expresses an HIV antigen or induces a viral specific immune response. Thus, Paoletti fails to motivate the selection of the claimed fowlpox vector encoding Gag and/or Pol over the various other vectors and HIV antigens encompassed by Paoletti.

Further, prior to the filing of applicants' application there was considerable uncertainty in the art regarding fowlpox vectors. For example, attached are copies of Paoletti, Proc. Natl. Acad. Sci. U.S.A, 93(21):11349-11353, 1996 and Taylor, Vaccine, 13(6):539-549, 1995. Both of these documents state that canarypox viruses have shown to be 100 times more efficient than a comparable fowlpox vector in inducing protective immunity (Paoletti, page 11350, second full paragraph and Taylor, page 539, passage bridging columns one and two). Accordingly, at the time of filing, one of ordinary skill in the art would not be motivated to select the claimed fowlpox virus vector from Paoletti.

In addition, Paoletti and Ramshaw do not provide any reasonable expectation that the claimed viral construct would be effective in inducing, enhancing or otherwise stimulating an immune response. The Examiner appears to consider that the prior art provides a general and broad expectation that co-expression of a cytokine with an antigen would enhance the immune response to an HIV antigen of interest (page 3, lines 9 et seq of the Office Action). The Examiner, however, has failed to identify why the combination of Paoletti and Ramshaw would have provided a reasonable expectation of success. Specifically, prior to the present invention, there had been no studies of co-expression (fowlpox-antigen-cytokine) in primates. As fowlpox poorly infects mammalian cells, there was a considerable risk that the vector would not work in primates. Further, at the time of filing, there was considerable uncertainty whether vectors which had shown to be immunogenic in mice would be immunogenic in primates. The technical data provided in the subject specification shows a vaccine construct according to the claims that is effective both in terms of its immunogenicity in primates and safety in primates. Without applicants' specification that provides proof of the effectiveness of the claimed viral construct, one of ordinary skill in the art would not be motivated to combine these specific references in the claimed manner to obtain a successful viral construct.

Accordingly, the Examiner has failed to provide the specific motivation required to combine the references. Further, the Examiner has failed to show that even if one of ordinary skill in the art were to have combined the disclosures of these references, they would have arrived at the claimed invention absent applicants' disclosure. Consequently, the rejections of claims 1, 17-19 and 38, should be withdrawn.

Claims 24, 25, 30 and 31 stand rejected under 35 USC 112, first paragraph, as not being enabled by the specification. In the interest of expediting prosecution, claims 24, 25, 30 and 31, have been cancelled without prejudice. Accordingly, this rejection is now moot.

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. **229752001400**.

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Respectfully submitted,

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Biological and immunogenic properties of a canarypox–rabies recombinant, ALVAC-RG (vCP65) in non-avian species

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A canarypox-based (ALVAC) recombinant expressing the rabies G glycoprotein has been utilized to assess in vitro *and* in vivo *biological properties of the canarypox virus vector system.* In vitro *studies have shown that no replication of the virus can be detected on six human-derived cell lines, nor can the virus be readily adapted to replicate on non-avian cells. Expression of the rabies G can be detected on all cell lines analyzed in the absence of productive viral replication. Analysis of viral-specific DNA accumulation indicated that the block in the replication cycle in the human cell lines analyzed occurred prior to DNA replication. The exact nature of the block, however, remains unknown. The concept of using a non-replicating immunization vehicle has been demonstrated through extensive* in vivo *studies in a range of species including non-human primates and humans. The results of such* in vivo *studies have exemplified the safety and immunogenicity of the ALVAC vaccine vector.*

Keywords: Poxvirus-based vaccines; canarypox virus (ALVAC); ALVAC-RG(vCP65); safety; immunogenicity

The development of naturally host-restricted avipox virus vectors capable of expressing extrinsic immunogens and inducing a protective immune response against lethal viral challenge in mammalian species has been described[1–5]. Fowlpox virus (FPV) and canarypox virus (CPV) are members of the avipox virus genus of the Orthopoxvirus family. Productive replication of avipox viruses is restricted to avian species[6]. Both FPV and CPV-rabies recombinants[1,2] express the rabies glycoprotein in tissue culture cells of non-avian origin without apparent replication of the vector virus. Inoculation of these recombinants into a range of non-avian species including mice, cats, and dogs demonstrated that the level of expression of the foreign gene product was sufficient to induce rabies-specific serum neutralizing antibodies and to protect against a lethal rabies virus challenge.

Potency tests in mice indicated that a CPV vector expressing the rabies glycoprotein was 100-fold more efficacious than an FPV-based vector and that the protective efficacy of a host-restricted CPV-rabies vector was similar to that of a replication-competent vaccinia virus vector containing the rabies G gene in the thymidine kinase locus[2]. Further, both replication competent VV-measles recombinants and a host-restricted CPV-measles recombinant induced similar levels of measles virus neutralizing antibody and protection against experimental canine distemper virus challenge in dogs[3].

Additional studies have shown that the utility of avipox vectors as immunizing agents in non-avian species is not limited to the rabies glycoprotein or measles virus immunogens. Vaccination of cats with an ALVAC-based recombinant expressing the feline leukemia virus (FeLV) A subtype Env and Gag proteins protects against the development of persistent viremia following FeLV challenge exposure[7]. ALVAC recombinants expressing immunogens from Japanese encephalitis virus (JEV) have also been shown to protect mice against a lethal JEV challenge[8]. Safety and immunogenicity studies in horses utilizing an ALVAC recombinant expressing the hemagglutinin glycoproteins from the A1 and A2 serotypes of equine influenza virus demonstrated the induction of type specific hemagglutination-inhibiting antibodies and protection against an A2 epizootic[4]. An ALVAC-based recombinant expressing the HIV-1 envelope glycoprotein has recently been shown to induce HIV-specific antibody and cytotoxic T-lymphocyte responses in mice[9]. These examples of ALVAC recombinants expressing immunogens from a variety of viral pathogens indicate the general utility of ALVAC-based

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recombinant viruses as immunization vehicles in a variety of mammalian species.

Avipox virus-based vectors theoretically provide significant safety advantages in light of their inability to productively replicate in non-avian species. Such a vaccine vector should not allow dissemination within the vaccinated individual, contact transmission to non-vaccinated individuals, or general contamination of the environment. The concept of utilizing a replication-restricted vaccine vector, ALVAC-RG, in humans has recently been assessed with promising results. The experimental vaccine was well tolerated and induced protective levels of rabies-neutralizing antibodies[10, 11].

These studies in target species have provided a database which has indicated that ALVAC-based recombinants may have significant advantages as vector-based vaccines. It therefore was critical to rigorously evaluate the safety characteristics of ALVAC-based recombinants in laboratory animals and to establish the innocuity and immunogenicity of the vector in both non-human primates and target species. This report describes *in vitro* and *in vivo* studies designed to explore the basis of host restriction, safety, and immunizing potential of the ALVAC-based vector.

MATERIALS AND METHODS

Cells and viruses

Viral amplifications and plaque titrations were performed on primary chicken embryo fibroblast (CEF) cells from 10 to 11 day embryos of SPF origin. Titrations were also performed as a microtiter assay on the quail QT35 cell line[12] and titers determined by the method of Karber[13].

The origin of other cells used in this study is as follows: (1) VERO cells (ATCC No. CCL81) are a line derived from African Green Monkey kidney; (2) MRC-5 (ATCC No. CCL171) are of human embryonic lung origin; (3) HNK are human neonatal kidney cells subcultured for less than five passages (Whittaker BioProducts, Inc., Walkersville, MD (Cat. No. 70-151)); (4) HEL 299 are human embryonic lung cells (ATCC No. CCL137); (5) WISH are of human amnion origin (ATCC No. CCL25); (6) DETROIT 532 are of human foreskin (Down's Syndrome) origin (ATCC No. CCL54); (7) JT-1 is a human lymphoblastoid cell line transformed with Epstein-Barr virus as described in Rickinson *et al.*[14]

The canarypox virus strain from which ALVAC was derived was isolated from a pox lesion on an infected canary. The virus was first isolated at the Rentschler Bakteriologishes Institut, Lauphein, Wurtemberg, Germany, where it was attenuated by 200 serial passages in CEFs. This attenuated strain (Kanapox) obtained from Rhone Merieux is licensed as a vaccine for canaries in France. At Virogenetics, the virus was subjected to four successive rounds of plaque purification under agarose. One plaque isolate, designated ALVAC, was selected for amplification and used in these studies.

Development of recombinant ALVAC-RG (vCP65)

The canarypox rabies recombinant was derived by methods previously described[1, 15, 16]. A unique insertion locus was defined at a *Bgl*II site within an 880 bp *Pvu*II fragment of CPV genomic DNA. The DNA sequence of this fragment was determined and the open reading frame (ORF) designated as C5 defined. Deletion of the entire C5 ORF was made by standard molecular biological procedures[17, 18] without interruption of neighboring ORFs. The C5 ORF was replaced by *Hind*III, *Sma*I and *Eco*R1 insertion sites followed by translation termination codons and early vaccinia virus transcription termination signals[19].

The ERA strain rabies glycoprotein cDNA[20, 21] linked to the early/late vaccinia virus H6 promoter[1, 16, 22] was inserted at the *Sma*I site. The resulting plasmid, pRW838, was transfected into ALVAC-infected primary CEF cells using the calcium phosphate precipitation method[15]. Plaques were selected on the basis of DNA hybridization to a rabies G-specific radiolabelled probe and subjected to sequential rounds of plaque purification. A representative plaque was then amplified and designated ALVAC-RG with the laboratory designation of vCP65.

Inoculation of non-avian cells with ALVAC-RG

A variety of human cell substrates, MRC-5, HNK, HEL, DETROIT-532, WISH and JT-1, were inoculated with ALVAC-RG and analyzed for expression of the rabies G gene, and accumulation of viral-specific DNA. Primary CEF cells were included as a permissive substrate.

Viral DNA accumulation

Sixty millimetre dishes containing two million cells of each cell type under test were inoculated with ALVAC at a multiplicity of infection (MOI) of 5 p.f.u. per cell. After an adsorption period of 1 h at 37°C, the inoculum was removed, the monolayer washed twice to remove unadsorbed virus and the infected monolayer refed with 5 ml of Eagle's Minimal Essential Medium (EMEM) + 2% Newborn Calf Serum (NCS). Cells from one dish were harvested at t_0 and the remaining dishes were incubated, in the presence or absence of 40 μg ml^{-1} of cytosine arabinoside (AraC; Sigma No. C6654), at 37°C for 72 h. Cells were collected and resuspended in 0.5 ml phosphate buffered saline (PBS) containing 40 mM EDTA and incubated for 5 min at 37°C. An equal volume of 1.5% agarose containing 120 mM EDTA, prewarmed to 42°C, was gently mixed with the cell suspension and transferred to an agarose plug mold. After solidification, the agarose plugs were removed and incubated for 12–16 h at 50°C in a volume of lysis buffer (1% sarkosyl, 100 μg ml^{-1} proteinase K, 10 mM Tris HCl pH 7.5, 200 mM EDTA) sufficient to cover the plug. The lysis buffer was then replaced with 5 ml 0.5×TBE (44.5 mM Tris borate, 44.5 mM boric acid, 0.5 mM EDTA) and equilibrated at 4°C for 6 h with 3 changes of 0.5×TBE buffer. The viral DNA within the plug was fractionated from cellular nucleic acid using a BIO-RAD CHEF-DR II pulse field electrophoresis system (180 V/20 h/15°C) in 0.5×TBE with a ramp time of 50–90 s, using lambda DNA as molecular weight standards. The viral DNA band was first visualized by staining with EtBr, then transferred to a nitrocellulose membrane and probed with a radiolabelled probe prepared from purified canarypox genomic DNA.

Analysis of expression of rabies G gene

Immunoprecipitation analysis was performed from a radiolabelled lysate of each cell line infected with ALVAC or ALVAC-RG (vCP65) as described in Tartaglia *et al.*[18] using a rabies glycoprotein-specific monoclonal antibody designated 24-3F10.

Time course study

MRC-5 and CEF monolayers were inoculated with 10 pfu/cell of ALVAC or ALVAC-RG (vCP65) at 37°C for 60 min. The inoculum was removed, the monolayer washed twice, and the medium replaced. At 1, 3, 5, 9, 13, 17, and 25 h post infection, the culture was labelled for 1 h by the addition of methionine-free medium containing 25 μCi ml^{-1} of ^{35}S-methionine (DuPont NEN; 1140 Ci mmol^{-1}). Infected cells were scraped from the culture dishes, collected by centrifugation, washed twice with PBS and lysed by the addition of 2 ml of Buffer A (18). Infected cell lysates were analyzed for expression of the rabies G gene by immunoprecipitation as described in Tartaglia *et al.*[18]

Safety studies in laboratory animals

Groups of rabbits (New Zealand white ESD), guinea pigs (Dunkin-Hartley, Libeau) and mice (IFFA Credo, Les Oncins, France) were inoculated with ALVAC-RG by a variety of routes as shown in *Table 1*. Animals were inspected daily for signs of reactogenicity and at the termination of the test at 14–21 days, animals were euthanized and tissue at the site of inoculation examined. To monitor neurovirulence, nine male OF$_1$ mice were anesthetized and injected by the i.c. route with ALVAC-RG (vCP65). Three mice were inoculated with an uninfected cell extract. Three inoculated and one control mice were sacrificed on days 1, 3 and 6 post-inoculation. Brains were fixed *in situ* by immersing the opened skull in a solution of buffered formalin. After processing, 5 sections were made and stained with gallocyanin/phloxine. The sections involved the following levels: A: corpus striatum, B: infundibulum, C: pedunculi cerebri, D: pons and E: cerebellum.

Comparison of virulence of Kanapox virus (Rentschler strain of CPV) and ALVAC-RG in canary birds

Canary birds certified to have not been immunized with canarypox virus were obtained from PIC Grains (Vignouse sur Barangeon, France). Birds were inoculated with 5.0 or 7.0 $\log_{10}$ p.f.u. of either Kanapox (Rentschler strain of CPV) or ALVAC-RG (vCP65) by smearing 50 μl of a 1:1 mixture of virus suspension and glycerin on a 0.5 cm^2 area from which the feathers had been removed on the back of each bird. Birds were monitored on a daily basis for one month postinoculation with weighing at 2–3 day intervals.

Table 1 Safety studies in laboratory animals: schedule of inoculation by different routes

Species	Virus	Dose	Route	Volume	Sites
Rabbit	ALVAC	5.7[a]	i.c.	0.1 ml	1
	ALVAC-RG	5.7	i.c.	0.1 ml	1
	ALVAC-RG	6.3	i.d.	0.2 ml	5
	ALVAC-RG	8.0	s.c.	9.0 ml	1
Guinea pig	ALVAC-RG	6.0	i.d.	0.1 ml	5
	ALVAC-RG	7.3	s.c.	2.0 ml	1
Mice	ALVAC-RG	5.7	i.d.	0.05 ml	5
	ALVAC-RG	6.7	s.c.	0.5 ml	1
	ALVAC-RG	6.0	i.c.	0.05 ml	1

[a]Inoculum dose expressed as $\log_{10}$TCID$_{50}$

Inoculation of ALVAC-RG into the skin of canary birds and mice

Female OF$_1$ mice were injected by the i.d. route in each ear pinna with 5.0 $\log_{10}$ TCID$_{50}$ of ALVAC-RG (vCP65) in 20 μl. Canary birds received an equivalent dose mixed with glycerin and smeared on a 1.0 cm^2 area of skin on the back from which feathers had been removed. At time intervals, animals were sacrificed and the skin surrounding the site of inoculation was dissected, homogenized in medium 199/Ham F10 plus 2% FCS, and stored at −70°C. Mouse specimens consisted of the entire skin covering the dorsal face of the ear pinna. Homogenates were thawed, sonicated, centrifuged, diluted 1:100 to avoid toxicity, and titrated in serial dilutions in QT35 cells.

Immunogenicity and safety studies in primate species

Three species of non-human primate, rhesus macaques, chimpanzees and squirrel monkeys (*Saimiri sciureus*) were inoculated with ALVAC-RG as shown in *Table 2*. The study in squirrel monkeys also addressed the questions of the ability to re-isolate virus after inoculation by a variety of routes and the immune response to ALVAC-RG in the face of pre-existing ALVAC immunity. In this study, three groups of four squirrel monkeys were inoculated with one of three viruses: (a) ALVAC, the parental canarypox virus; (b) ALVAC-RG (vCP65); or (c) ALVAC-FL (vCP37), a canarypox recombinant expressing the envelope glycoprotein of FeLV (Tartaglia *et al.*, unpublished data). Inoculations were performed under ketamine anesthesia. Each animal received at the same time: (1) 20 μl instilled on the surface of the right eye without scarification; (2) 100 μl as several droplets in the mouth; (3) 100 μl in each of two i.d. injection sites in the shaven skin of the external face of the right arm; and (4) 100 μl in the anterior muscle of the right thigh. In each group, two animals received 5.0 $\log_{10}$ p.f.u. and two animals received 7.0 $\log_{10}$ p.f.u. of the appropriate virus. Virus isolation was attempted from the site of inoculation for 11 days post-inoculation and all monkeys were monitored for adverse reactions. Six months after the initial inoculation, selected animals from each group plus one canarypox naive animal were inoculated with ALVAC-RG (vCP65) as described in *Table 2*. All animals were monitored for adverse reactions to vaccination and sera analyzed for the presence of anti-rabies antibodies[24].

RESULTS

Derivation of ALVAC-RG (vCP65)

The strategy used to develop FPV[1, 16, 23] and CPV[2, 3] recombinants involved insertion of the foreign gene at a unique restriction site within an ORF previously defined as nonessential. No attempt was made to precisely delete the interrupted ORF. In the generation of ALVAC-RG (vCP65), an insertion plasmid containing the H6/rabies G expression cassette was constructed

Table 2 Schedule of inoculation of primate species with ALVAC-RG (vCP65)

Species	Designation	Dose[a]	Route	Previous inoculations	Booster dose, interval
Rhesus macaque	177 and 186	7.7	s.c.	none	7.0, 100 days
	178	7.0	s.c.	none	none
	182	7.0	i.m.	none	none
	179	6.0	s.c.	none	none
	183	6.0	i.m.	none	none
	180	5.0	s.c.	none	none
	184	5.0	i.m.	none	none
Chimpanzee	431	7.0	i.m.	none	7.0, 84 days
	457	7.0	s.c.	none	7.0, 84 days
Squirrel monkey	37, 53	6.5	s.c.	5.0, ALVAC-RG	180 days
	38, 54	6.5	s.c.	7.0, ALVAC-RG	180 days
	22, 51	6.5	s.c.	5.0, ALVAC	180 days
	39, 55	6.5	s.c.	5.0, ALVAC-FL[b]	180 days
	57	6.5	s.c.	none	none

[a]Virus dose expressed as $\log_{10}$ p.f.u. per ml
[b]A canarypox recombinant expressing the envelope glycoprotein of FeLV (Tartaglia *et al.*, unpublished data)

such that the flanking arms of the plasmid directed replacement of the non-essential ORF with the foreign gene. Insertion of the foreign gene was accomplished without altering neighboring ORFs and the generation of novel ORFs was precluded by engineering translational stop codons in all appropriate reading frames. The derived recombinant, ALVAC-RG (vCP65), was confirmed to contain the rabies G expression cassette in the correct C5 locus by Southern blot analysis, PCR analysis, and nucleotide sequence analysis (data not presented). Further, expression analyses by immunofluorescence and immunoprecipitation using a rabies G glycoprotein-specific monoclonal antibody confirmed the expression of the 66 kDa rabies glycoprotein on the surface of ALVAC-RG infected cells.

In vitro studies

Analysis of expression of the rabies G gene in avian and human derived cells. Prior results have indicated that ALVAC and derived recombinants do not productively replicate in a range of non-avian cell lines, including those derived from monkey, mouse, cat and human[18] (unpublished results). Additionally, in a similar study to that described in Taylor *et al.* [1] attempts to adapt ALVAC and ALVAC-RG (vCP65) to grow in two non-avian cell lines (MRC-5 and VERO) have failed[25]. Blind passages of both ALVAC and ALVAC-RG were performed in VERO, MRC-5 and primary CEF monolayers for 8 or 10 sequential passages of 7 days duration. While a 100-fold increase in viral titer was apparent in CEF cells after each passage in the series, after one passage in mammalian cells, the viral titer was lower than the residual input titer and titers fell below the level of detectability after two passages[25].

In order to establish that in the absence of productive viral replication the rabies glycoprotein (G) was expressed in the human derived cell lines, immunoprecipitation experiments were performed. The results of a representative analysis are shown in *Figure 1*. No specific immunoprecipitation products were detected in lysates derived from uninfected cells (lanes a, d and g) or cells infected with the parental ALVAC virus (lanes b, e and h). Immunoprecipitation of a 66 kDa protein by the rabies-specific monoclonal antibody was apparent from lysates derived from ALVAC-RG infected CEF, HNK and HEL cells (*Figure 1*, lanes c, f and i, respectively). This size is consistent with that described for SDS–PAGE of the rabies glycoprotein G[26].

In order to determine whether expression of the rabies G gene product would be maintained in human derived cells inoculated with ALVAC-RG (vCP65) in the absence of productive replication, a time course study was performed as described in Materials and Methods. Immunoprecipitation of the rabies G is shown in *Figures 2*a (CEF cells) and 2b (MRC-5 cells). Expression of the rabies G in both CEF and MRC-5 cells occurs as early as 1 h post-infection and continues undiminished under the control of the early/late H6 promoter throughout the labelling period of 24 h.

Analysis of viral specific DNA accumulation in human derived cells inoculated with ALVAC-RG (vCP65). In order to assess the temporal nature of the block in viral replication in human derived cells, the following experiment was performed. Permissive CEF cells and the six human derived cell lines were inoculated with ALVAC parental virus at an MOI of 5 pfu per cell in the presence or absence of AraC, an inhibitor of DNA replication, and the level of virus specific DNA accumulated at 72 h was assessed as described in Materials and Methods. *Figure 3* illustrates analysis of CEF, WISH and DETROIT 532 cells. In the permissive cell line, CEF (*Figure 3*; Panel B), no viral-specific DNA is seen in lane B1 (uninfected CEF cells), lane B2 (ALVAC-infected CEF cells at t_0) or lane B4 ALVAC infected CEF cells at 72 h post-infection in the presence of AraC). Viral specific DNA accumulation represented by a band at approximately 330 kbp is evident in ALVAC infected CEF cells incubated for 72 h in the absence of AraC (lane B3). No such accumulation is seen in the equivalent sample of ALVAC infected DETROIT-532 (lane A3) or WISH (lane C3) cells. Similar results were observed on analysis of ALVAC specific DNA accumulation in MRC-5, HEL, HNK and JT-1 infected cells (results not shown). Based on the conditions employed in these studies, the sensitivity of detection was determined as ≥ 125 genome equivalents. In further experiments, ^{3}H-thymidine incorporation into ALVAC-infected MRC-5 and CEF cells was monitored. These experiments indicated that while an increase in ^{3}H-thymidine incorporation occurred in CEF cells

following infection with ALVAC, in MRC-5 cells, ^{3}H-thymidine incorporation did not rise above basal levels (results not shown). The results indicate that under these conditions, no detectable ALVAC-specific DNA accumulation occurred in the human cell substrates and suggest that replication of ALVAC in these human cells is blocked prior to viral DNA synthesis.

In vivo safety studies with ALVAC-RG (vCP65)

Inoculation of laboratory animals. Previous experiments with CPV and FPV based recombinants in numerous species including mice, rabbits, rats, guinea pigs, cats, dogs, horses, cattle, and swine had demonstrated no adverse reactions upon inoculation by a variety of routes. It was important, however, to examine more stringently the safety profile of an ALVAC-based recombinant in mammalian species.

In a series of experiments described in Materials and Methods and *Table 1*, mice, guinea pigs and rabbits were inoculated with ALVAC-RG by a variety of routes and reactivity monitored. The results indicated that no reactivity was evident in any species following inoculation by the subcutaneous route. Similarly, there was no evidence of neurovirulence apparent in mice or rabbits inoculated with approximately 6.0 $\log_{10}$ $TCID_{50}$ of ALVAC-RG by the intracranial route. Ten rabbits inoculated in this manner showed no local or systemic adverse reactivities, exhibited normal weight gain and no lesions were found in the brain. In mice, in which histopathology was performed, there was no indication of encephalopathy caused by ALVAC-RG (vCP65) in the sections observed. Only very high doses of ALVAC were found to be lethal by i.c. inoculation in young adult or newborn mice further suggesting the lack of neurovirulence of the ALVAC virus[18].

After inoculation by the i.d. route, reactions were evident at the site of inoculation in mice, guinea pigs, and rabbits. In mice, the reactions consisted of small necroses, a few millimeters in diameter, at the site of inoculation. These were evident by 1 day post-

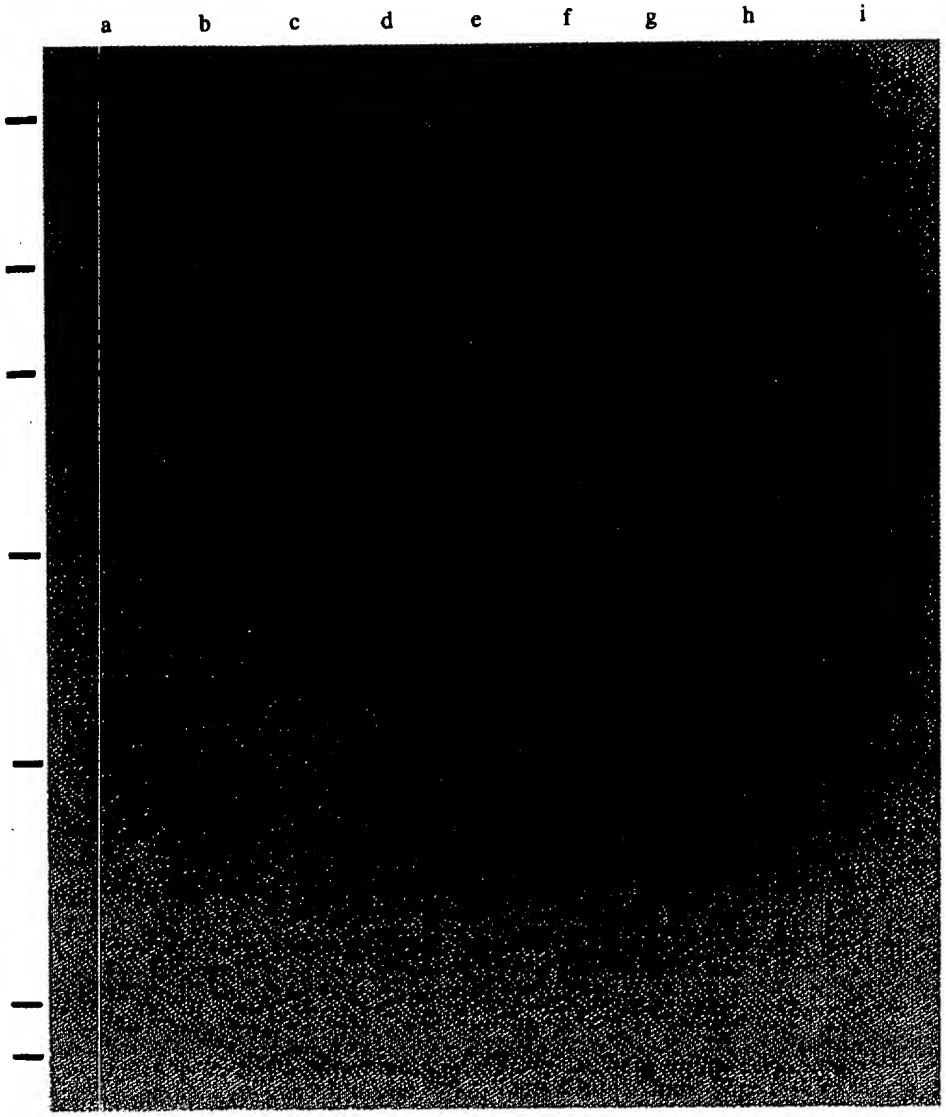

Figure 1 Immunoprecipitation analysis of expression of the rabies glycoprotein in avian and non-avian cells inoculated with ALVAC-RG (vCP65). Dishes of each cell line were inoculated at an input multiplicity of 10 p.f.u./cell with ALVAC or ALVAC-RG in the presence of ^{35}-S methionine as described in Ref. 18. Lanes a, d, g, uninfected cells; lanes b, e and h, ALVAC infected cells; lanes c, f and i, ALVAC-RG (vCP65) infected cells. Lanes a, b and c, CEF cells; lanes d, e and f, HNK cells; lanes g, h and i, HEL cells. Molecular weight markers are shown to the left of lane a and indicate migration distances for standard proteins with molecular weights (from the top) of 200, 97.4, 68, 43, 29, 18.4 and 14.3 kDa

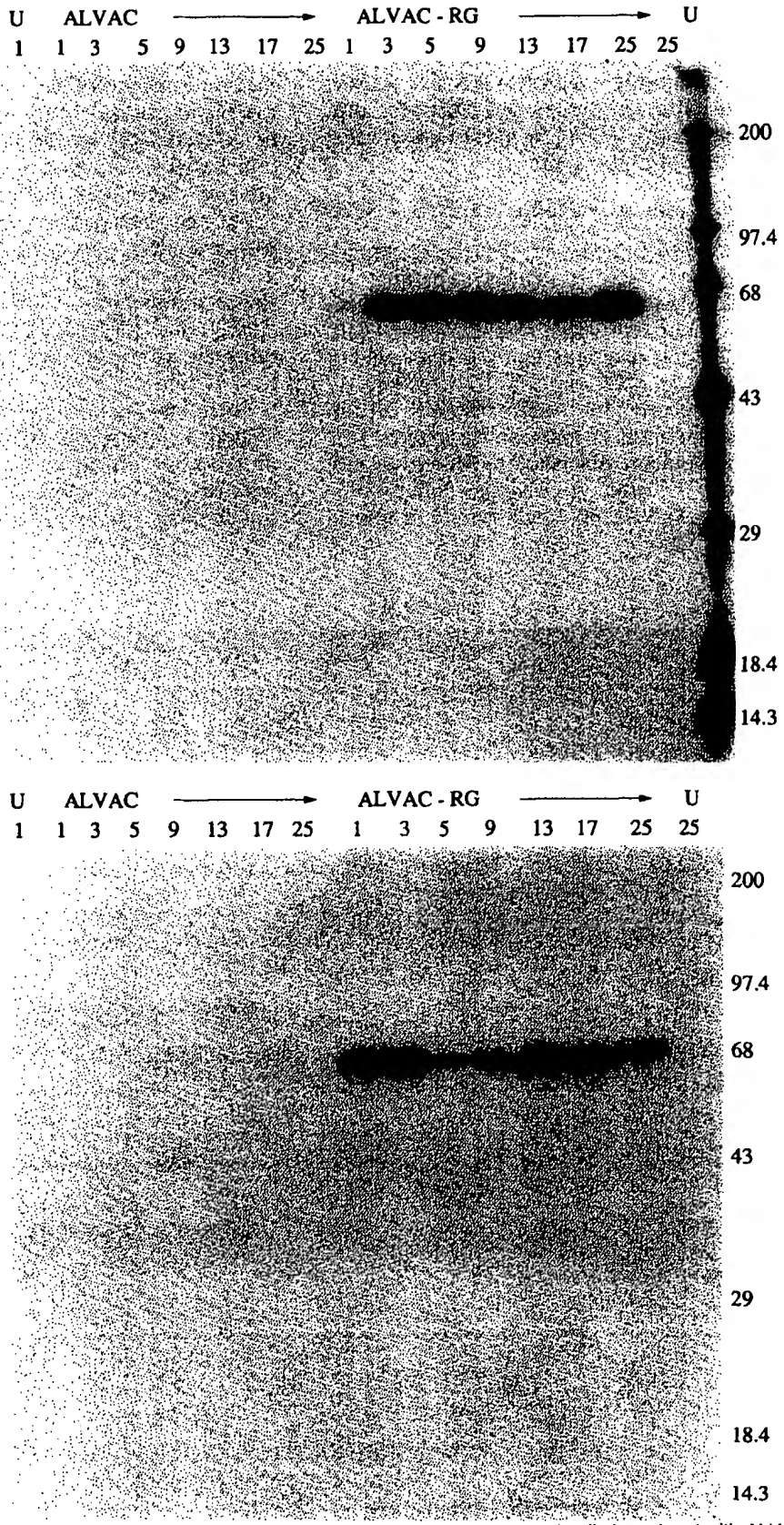

Figure 2 Time course of analysis of expression of the rabies G protein in avian and human-derived cells inoculated with ALVAC-RG. Dishes of CEF or MRC-5 cells were inoculated at an input multiplicity of 10 p.f.u. per cell with ALVAC or ALVAC-RG and labelled for 1 h with ^{35}S-methionine at appropriate times as described in Materials and Methods. Immunoprecipitation was performed as described previously[18]. Figure 2a illustrates analysis of CEF cells and 2b illustrates analysis of MRC-5 cells. U denotes an uninfected control cell lysate, ALVAC or ALVAC-RG indicates cells were infected with ALVAC parental virus or ALVAC-RG, respectively. Figures at the top of each lane indicate time of the labelling period in hours post-infection. Molecular weight markers are shown at the right of each figure (a and b) for migration of standard proteins as described in the legend to Figure 1

inoculation persisting approximately 7 days. In guinea pigs, there was a more inflammatory reaction which consisted of erythema, then a small pustule followed by necrosis. In rabbits, an inflammatory reaction was also seen which progressed to a small pustule with necrotic patches. In both guinea pigs and rabbits the dermal lesions were resolved by 21 days post-inoculation. In a similar experiment, serial dilutions of ALVAC-RG (vCP65) and KANAPOX, the original vaccinal strain of canarypox from which ALVAC was derived, were inoculated by the i.d. route into rabbits and the reactogenicity assessed. Both ALVAC-RG (vCP65) and Kanapox induced some erythema and edema with slight necrosis at the inoculation site indicating the reactogenicity was similar with the parental strain and ALVAC-RG recombinant virus. The reactivity was dose related being most pronounced with the undiluted preparation which contained 6.2 $\log_{10}$ $TCID_{50}$ per ml. It should be noted that viral preparations used in these experiments were not gradient purified and the presence of cellular components may have contributed to the reactogenicity seen following injection by the i.d. route.

Inoculation of canary birds with ALVAC-RG. In order to confirm that the virulence of the parental strain had not been altered by deletion of the C5 ORF and insertion of a heterologous coding sequence (the rabies G gene), reactogenicity of Kanapox and ALVAC-RG (vCP65) was compared in canary birds. No deaths occurred in any of the birds and body weights varied within physiological limits throughout the experimental period. One bird in each group inoculated with 5.0 $\log_{10}$ p.f.u. of ALVAC-RG or Kanapox showed mild inflammation at the application site during the second week post-inoculation with redness and some swelling. All birds inoculated with 7.0 $\log_{10}$ p.f.u. of either virus developed a typical pox-like take on day 5 with inflammation, swelling, a small pock, and in one bird inoculated with ALVAC-RG (vCP65), a patch of

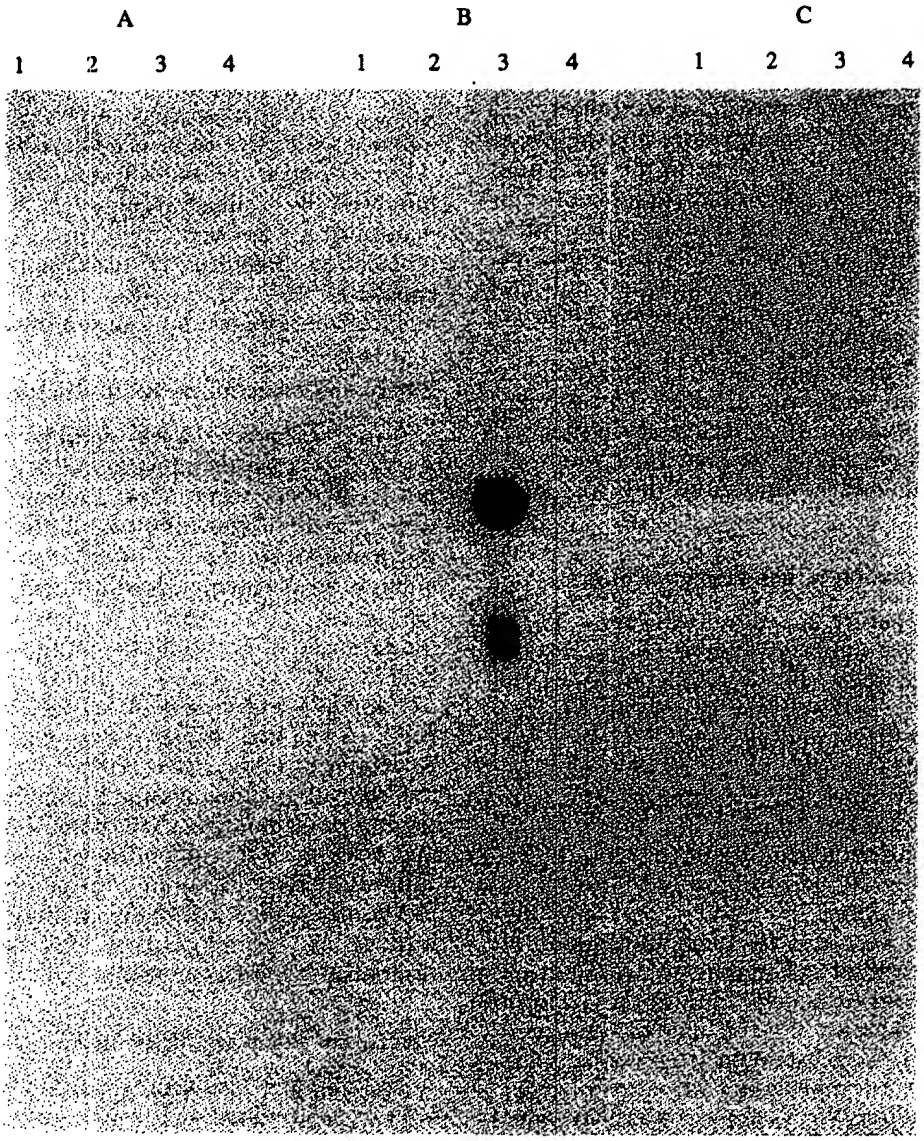

Figure 3 Analyses of viral-specific DNA accumulation in avian and human derived cell lines inoculated with ALVAC. Dishes of each cell line were inoculated and processed as described in Materials and Methods. Panel A, DETROIT 532 cells; Panel B, CEF cells, Panel C, WISH cells. In each panel, lane 1 represents uninoculated cells, lane 2 represents ALVAC-infected cells harvested at zero time, lane 3 represents ALVAC-infected cells harvested at 72 h and lane 4 represents ALVAC-infected cells incubated in the presence of 40 $\mu g\ ml^{-1}$ of AraC and harvested at 72 h

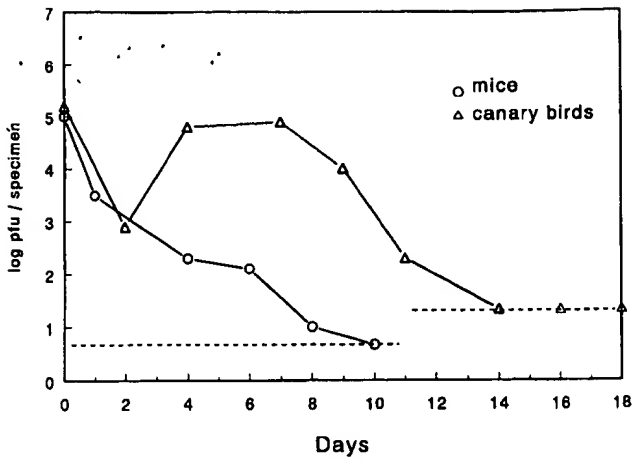

Figure 4 Analysis of virus isolation from skin of canaries and mice inoculated with ALVAC-RG. Mice and canaries were inoculated with ALVAC-RG by the i.d. route as described in Materials and Methods. At time intervals after inoculation animals and birds were sacrificed and the skin surrounding the site of inoculation was dissected, homogenized and assayed for the presence of infectious virus by titration on QT35 cells

necrosis. All lesions remained localized and were resolved by 21 days post-inoculation.

A further experiment was performed to follow the fate of inoculated virus in canary birds and in mice. Mice were inoculated by the i.d. route in the ear pinna and canary birds by smearing virus onto an area of skin on the back of the bird from which feathers had been plucked. The results of virus isolation from the area of inoculation are shown in *Figure 4*. The results indicate that in canary birds, there was an initial eclipse phase of 2 days after which an increase in infectious virus was observed for up to 7 days. Viral recovery then declined and after 14 days no virus was detected. In contrast, from day 0 the amount of virus recovered from mice progressively declined until reaching the limit of detection (20 p.f.u. per sample) on day 10 after inoculation.

In vivo studies: inoculation of non-human primates

Safety and immunogenicity in squirrel monkeys. Three groups of four squirrel monkeys (*Saimiri sciureus*) were inoculated as described in MATERIALS AND METHODS and *Table 2*, and monitored for reactogenicity as well as immune response. Animals received either ALVAC parental virus (animal Nos 22, 51, 36, and 52), ALVAC-RG (vCP65 animal) (Nos 37, 53, 38, and 54) or a canarypox recombinant expressing an Env gene product derived from an endogenous FeLV provirus (animal Nos 39, 55, 40, and 56). The initial inoculation was performed by ocular, oral and i.d. routes. No reactions were seen following inoculation of the three viruses except for minor skin lesions following i.d. inoculation of approximately 7.0 $\log_{10}$ p.f.u. Both body weight and temperature of all animals remained within normal limits. Virus isolation from ocular fluid, saliva and the site of i.d. inoculation was attempted for 11 days post-inoculation. Virus recovery was achievable from the inoculation site for 2 (6/6 animals) to 4 days (2/6 animals) following the i.d. administration of 7.0 $\log_{10}$ p.f.u. but not 5.0 $\log_{10}$ p.f.u. of all viruses. Virus was not recoverable from eye secretions or saliva at any timepoint (results not shown).

Analysis of post-inoculation sera by ELISA indicated that all animals inoculated with either ALVAC or an ALVAC-based recombinant developed a serological response to ALVAC (results not shown). All four animals inoculated with ALVAC-RG (vCP65) (animal Nos 37, 53, 38, and 54) developed rabies virus neutralizing antibody (*Table 3*), the level of which at 28 days was well above that considered to be a satisfactory response to rabies vaccination. It should be noted that 0.5 International Units, or a titer of approximately 1:16 is considered by the WHO to be the acceptable minimal response to rabies vaccination[27]. Six months after the primary inoculation, four monkeys which received ALVAC-RG (vCP65) (37, 53, 38, and 54), two monkeys which received ALVAC (22, 51), two monkeys which received an ALVAC recombinant expressing the FeLV env gene (39, 55), and one naive monkey (57) were inoculated with 6.5 $\log_{10}$ p.f.u. of ALVAC-RG (vCP65) by the s.c. route to monitor the immune response in the face of pre-existing ALVAC immunity. There were no adverse reactions to re-inoculation in any of the animals. At 28 weeks all previously inoculated animals showed some low level of canarypox ELISA antibody which was boosted 3–7 days after reinoculation (results not shown). Assessment of levels of anti-rabies antibody in sera of these animals is shown in *Table 3*. The four animals with prior exposure to ALVAC (22 and 51) or ALVAC-FeLV (39 and 55) and the naive animal (57) mounted a primary response with rabies virus neutralizing antibody present 7–11 days post-inoculation. Significantly, the four monkeys with prior exposure to ALVAC-RG (vCP65) showed an anamnestic response by 7 days post-inoculation.

Safety and immunogenicity in Rhesus macaques

Two macaques were initially inoculated with ALVAC-RG as described in *Table 2* by the s.c. route. No local or systemic adverse reactions to inoculation were noted. After 100 days, these animals were reinoculated by the s.c. route and an additional six animals were inoculated with a range of doses by the i.m. or s.c. routes. Sera of animals were monitored for the presence of anti-rabies neutralizing antibody in the RFFIT-test[24] and results are shown in *Table 4*. Animals 177 and 186 receiving ALVAC-RG (vCP65) by the s.c. route developed rabies virus neutralizing antibody detectable at 11 days post primary inoculation. Levels of antibody above the minimal acceptable level[27] were still present at 3 months when animals were re-inoculated and both animals responded with an increase in titer. Equivalent responses were obtained by either the s.c. or i.m. routes with a dose of either 7.0 or 6.0 $\log_{10}$ p.f.u. At a dose of 5.0 $\log_{10}$ p.f.u. only one animal (180) responded by the s.c. route.

Safety and immunogenicity studies in chimpanzees

Two chimpanzees were inoculated by the i.m. (animal 431) or s.c. (animal 457) routes with 7.0 $\log_{10}$ p.f.u. of ALVAC-RG (vCP65). At 12 weeks, both animals were re-inoculated in an identical manner. No local or systemic adverse reactions to inoculation were noted in either animal. Serological results are shown in *Table 5*. Both chimpanzees responded with the induction of

Table 3 Response of squirrel monkeys with prior exposure to ALVAC or ALVAC-based recombinants to inoculation with ALVAC-RG

Animal number	First inoc.	RFFIT titer at day post-inoculation[a]					
		0	7	28	180[d]	187	201
22	ALVAC	—	—	—	<16	<16	200
51	ALVAC	—	—	—	<16	50	158
39	ALVAC-FL	—	—	—	<16	50	158
55	ALVAC-FL	—	—	—	<16	50	126
37[b]	ALVAC-RG	<16	<16	1000	<16	1580	3160
53[b]	ALVAC-RG	<16	16	158	<16	3980	3980
38[c]	ALVAC-RG	<16	316	1000	<50	1580	3980
54[c]	ALVAC-RG	<16	250	1580	<50	3980	10000
57	None	—	—	—	<16	50	500

[a]Sera tested in an RFFI Test (Ref. 24). Titer expressed as eciprocal of the highest dilution showing complete inhibition of fluorescence.
[b]Animals 37 and 53 were inoculated with 5.0 $\log_{10}$ p.f.u. as described in Table 2
[c]Animals 38 and 54 were inoculated with 7.0 $\log_{10}$ p.f.u. as described in Table 2
[d]At day 180, all animals were inoculated with 6.5 $\log_{10}$ p.f.u. of ALVAC-RG by the subcutaneous route

rabies virus neutralizing antibody at 2–4 weeks post-inoculation and antibody titers were significantly boosted after the second inoculation at 12 weeks.

DISCUSSION

The studies described in this communication were conceived to evaluate, in some detail, the biological and immunological properties of ALVAC and derivative recombinants in non-avian species. The results provide a safety profile for the ALVAC vaccine vector and illustrate the utility of ALVAC as a general immunization vehicle in non-avian species.

Failure to demonstrate replication of ALVAC or ALVAC-based recombinants has been demonstrated on tissue culture cells of murine and feline origin (unpublished data). Further, no evidence for viral replication has been obtained following inoculation of ALVAC on a variety of human or monkey-derived tissue culture systems[18] and the inability to adapt the virus to growth on human or monkey-derived cell lines has been confirmed by serial blind passage of both ALVAC and ALVAC-RG (vCP65)[25].

Table 4 Serological response following inoculation of Rhesus macaques with ALVAC-RG

Animal No. /dose	Route 1°/2°	RFFIT titer at days post-inoculation								
		0	6	11	35	99[a]	101	105	114	128
177/7.7	s.c./s.c.[b]	—	—	16[d]	32	64	32	512	512	256
186/7.7	s.c./s.c.	—	—	128	512	256	256	512	512	256
178/7.0	s.c.					—	—	—	64	64
182/7.0	i.m.[c]					—	—	—	32	64
179/6.0	s.c.					—	—	—	64	32
183/6.0	i.m.					—	—	—	128	128
180/5.0	s.c.					—	—	—	32	32
184/5.0	i.m.					—	—	—	—	—

[a]Day of re-inoculation
[b]Subcutaneous route
[c]Intramuscular route
[d]Titers expressed as reciprocal of last dilution showing inhibition of fluorescence in RFFI test[24]

Table 5 Serological response of chimpanzees to inoculation with ALVAC-RG

Animal No. /route	RFFIT titer at weeks post-inoculation									
	0	1	2	4	8	12[a]	13	15	20	26
431/i.m.	<8[b]	<8	8	16	16	16	128	256	64	32
457/s.c.	<8	<8	32	32	32	8	128	512	128	128

Animals were inoculated with 7.0 $\log_{10}$ p.f.u. of ALVAC-RG by the indicated route and re-inoculated in the same manner 12 weeks later
[a]Time of reinoculation
[b]Titer expressed as reciprocal of last dilution showing inhibition of fluorescence in an RFFI test[24]

On human-derived cell cultures, no accumulation of ALVAC-specific viral DNA was demonstrated suggesting that the block to viral replication in these cell substrates occurs early in the replication cycle prior to viral DNA replication. Similar analyses performed with ALVAC-infected VERO cells have demonstrated low, but detectable, levels of accumulated ALVAC-specific DNA (data not shown). Somogyi *et al.*[28] have recently shown that in MRC-5 cells infected with fowlpox virus, both viral DNA replication and some late viral protein synthesis can be detected, albeit at reduced levels. The block in avipox productive replication in mammalian cells may vary, not only for different cell types, but also for the different avipox viruses. While the details of the molecular events responsible for the block to viral replication in non-avian species remain to be elucidated, it is significant that the expression of at least some avipox virus genes, and of appropriately regulated extrinsic immunogens, occurs in all non-avian tissue cultures tested. Additionally, when the time course of expression of the rabies G gene was monitored in these cells, it was evident that expression could be detected continuously from 1 to 25 h post-infection when the experiment was terminated (*Figure 2*).

Previous *in vivo* studies in a variety of species including mice, cats, and dogs[2, 3] had shown no reactogenicity following inoculation of a CPV recombinant. A number of laboratory animals were inoculated with ALVAC or ALVAC-RG (vCP65) to extend these results. Safety studies performed in laboratory animals via the s.c. and i.m. routes indicated no reactogenicity. Similarly, inoculation of mice and rabbits by the i.c. route showed no evidence of neurovirulence. This is also supported by data of LD_{50} values by i.c. inoculation of young or newborn mice[18]. Further, no adverse reactions have been observed upon inoculation of immunodeficient mice[18].

Inoculation of rabbits with high doses of CPV by the i.d. route resulted in the formation of poxvirus-like lesions. In related experiments not reported here, lesions were induced on rabbits by i.d. inoculation of 8.0 $\log_{10}$ p.f.u. of ALVAC. When the dose was reduced to 7.0 and 6.0 $\log_{10}$ p.f.u., minimal reactogenicity was apparent. Similarly, skin lesions were evident at the site of i.d. inoculation of ALVAC and derived recombinants in squirrel monkeys but only sporadic virus recovery was possible through 4 days post inoculation. The formation of a lesion at the site of i.d. inoculation may be a cytotoxic phenomenon due to expression of early viral functions or may be linked to the presence of cellular components in the inoculum. Reactogenicity by the i.d. route was not related to altered pathogenicity following insertion of a foreign gene since an equivalent effect was seen with the parental canarypox vaccine strain, Kanapox.

Three non-human primate species were inoculated with ALVAC-RG (vCP65) to monitor safety and immunogenicity. No adverse signs of infection or disease were seen in the squirrel monkeys, macaques, or chimpanzees following inoculation by a variety of routes. All three species responded with significant levels of rabies virus neutralizing antibody which were boosted after a second inoculation. Significantly, squirrel monkeys with a history of prior exposure to CPV or CPV recombinants did not show a diminution of response when inoculated with ALVAC-RG (vCP65). These monkeys have since been inoculated with a third ALVAC recombinant expressing the measles virus fusion and hemagglutinin glycoproteins and have responded with protective levels of measles virus HI antibody comparable to that induced in naive animals (unpublished data). These results indicate that prior exposure to ALVAC recombinants should not preclude subsequent vaccinations with a novel ALVAC recombinant. In addition, it should be noted that four of the monkeys (22, 37, 38 and 39) had also received vaccinia virus three months before inoculation with ALVAC or ALVAC-based recombinants. The fact that the rabies-specific immune response was not diminished in these animals may indicate that in humans, prior immunity to vaccinia virus may not limit use of an ALVAC-based recombinant vaccine. The concept of using a non-replicating vector system in humans has been demonstrated in Phase 1 clinical trials with the ALVAC-RG (vCP65) recombinant virus. Volunteers inoculated with ALVAC-RG (vCP65) demonstrated significant immune responses to the extrinsic immunogen in the absence of unacceptable local or systemic reactions to vaccination[10, 11].

Practical issues of utilizing ALVAC-based recombinants for specific veterinary applications have been addressed in the target species. In a duration of immunity study, dogs inoculated with a single dose (6.7 $\log_{10}$ $TCID_{50}$) of ALVAC-RG (vCP65) were protected against a lethal challenge with rabies virus at 36 months post-inoculation (manuscript in preparation). Other unpublished studies have provided evidence that these vector systems may be useful in the presence of maternally derived antibodies (manuscript in preparation). The safety and immunogenicity profile of ALVAC-based recombinants suggests a strong potential for ALVAC as a generic immunization vehicle in other veterinary as well as human applications.

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Colloquium Paper

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Applications of pox virus vectors to vaccination: An update

(vaccinia/fowlpox/canarypox/NYVAC vaccinia)

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ABSTRACT **Recombinant pox viruses have been generated for vaccination against heterologous pathogens. Amongst these, the following are notable examples. (*i*) The engineering of the Copenhagen strain of vaccinia virus to express the rabies virus glycoprotein. When applied in baits, this recombinant has been shown to vaccinate the red fox in Europe and raccoons in the United States, stemming the spread of rabies virus infection in the wild. (*ii*) A fowlpox-based recombinant expressing the Newcastle disease virus fusion and hemagglutinin glycoproteins has been shown to protect commercial broiler chickens for their lifetime when the vaccine was administered at 1 day of age, even in the presence of maternal immunity against either the Newcastle disease virus or the pox vector. (*iii*) Recombinants of canarypox virus, which is restricted for replication to avian species, have provided protection against rabies virus challenge in cats and dogs, against canine distemper virus, feline leukemia virus, and equine influenza virus disease. In humans, canarypox virus-based recombinants expressing antigens from rabies virus, Japanese encephalitis virus, and HIV have been shown to be safe and immunogenic. (*iv*) A highly attenuated vaccinia derivative, NYVAC, has been engineered to express antigens from both animal and human pathogens. Safety and immunogenicity of NYVAC-based recombinants expressing the rabies virus glycoprotein, a polyprotein from Japanese encephalitis virus, and seven antigens from *Plasmodium falciparum* have been demonstrated to be safe and immunogenic in early human vaccine studies.**

The notion that the work of Edward Jenner could be carried on after the successful global eradication of smallpox as a human infectious disease was provided by early descriptions of the engineering of vaccinia virus to express foreign genes (1, 2). Thus, by splicing genes from heterologous pathogens into the vaccinia virus vector one could immunize against that cognate pathogen. The 14 years since those publications were an exciting period where numerous strains of vaccinia were engineered to express a variety of antigens from a myriad of bacterial, viral, and parasitic pathogens with subsequent evaluation of the recombinants in both animal models as well as target species. Initial safety concerns of vaccinia virus vectors have been addressed by the use of highly attenuated replication-deficient strains of the virus as well as the engineering of host range-restricted pox viruses such as canarypox virus that, while restricted for productive replication to avian species, have been shown to effectively vaccinate nonavian targets. The initial studies on vaccinia virus were extended to other members of the pox virus family so as to provide species specific vectors. An example of this is the engineering of fowlpox-based vectors for use as recombinant vaccines in the poultry industry. Much information has been gained through this period and today some commercial success has been evidenced by the licensing of several products in the veterinary field. Today, in addition to continued work in this area for vaccines, pox virus-based vectors remain as eminent tools for studying the parameters of immune induction and new fields of endeavor are being investigated such as in cancer immunotherapy.

This paper will provide an update, albeit incomplete, of ongoing research with pox virus-based vectors.

Vaccinia-Rabies Glycoprotein G Recombinant

A vaccinia recombinant expressing the rabies virus glycoprotein was an early example of a successful pox virus vector useful in immunization (3). The vector was constructed by the insertion of the encoding cDNA for the rabies virus glycoprotein in the thymidine kinase locus of the Copenhagen strain of vaccinia virus. Disruption of the thymidine kinase locus allowed a biochemical selection of the recombinant as well as an attenuated phenotype to the vector. This recombinant has received a conditional commercial license in both Europe and in the United States.

The recombinant is administered as a live vaccine in baits for oral uptake by foxes in Europe and by raccoons in the United States. Extensive seeding of large geographic regions has provided field safety and efficacy. More recently, vaccine baits for controlling an epizootic of rabies in coyotes and grey fox in Texas has involved the seeding by air of more than 40,000 square miles with this recombinant vaccine.

Fowlpox Virus-Based Recombinants

The engineering of fowlpox virus-based vectors has direct application for recombinant vaccines in the poultry industry. Fowlpox virus is a pathogen in poultry. Attenuated fowlpox virus vaccines have been used for decades in the poultry industry to prevent wild-type virus infection. These attenuated fowlpox vaccine strains provide starting material for further construction of recombinant vaccines. The vector approach in poultry is confronted by issues similar to the general vaccine discipline and specifically to the vector approach. One such issue is how will preexisting maternal immunity influence the outcome of vaccination with a recombinant vector approach. In the poultry industry, this problem is generally twofold since the mother is immune to both the pathogen whose genes are to be expressed in the vector and to the fowlpox vector itself. The results of such a situation are detailed in ref. 5, where a fowlpox virus recombinant expressing the hemagglutinin neuraminidase and the fusion glycoproteins of Newcastle disease virus (NDV) are studied. A single inoculation in specific

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Abbreviations: NDV, Newcastle disease virus; JEV, Japanese encephalitis virus; PRV, Pseudorabies virus; FeLV, feline leukemia virus; CTL, cytotoxic T lymphocyte.

pathogen-free birds at 1 day of age provided hemagglutinin-inhibiting antibodies that were maintained for the 8-week test period, which is the lifespan of a commercial broiler. Protective immunity was demonstrated against a combined intramuscular velogenic NDV challenge and a respiratory NDV challenge. Significantly, vaccination of commercial broiler chickens that retained a level of maternal immunity against both NDV and the vector resisted a subsequent challenge against both a lethal intramuscular NDV challenge, as well as a virulent fowlpox virus challenge. However, the NDV-specific immune response was at a reduced level. A fowlpox virus recombinant expressing NDV glycoproteins has received commercial licensure in the United States.

Avipox Virus Vectors in Nonavian Species

Members of the *Avipox* genus such as fowlpox and canarypox are distinguished by their host restriction for replication to avian species. It was discovered that inoculation of avipox-based recombinants into mammalian cells resulted in expression of the foreign gene and that inoculation into mammals resulted in the induction of protective immunity (6, 7). This surprising finding provided a significant safety profile to these vectors. Immunization could be affected in the absence of productive replication while eliminating the potential for dissemination of the vector within the vaccinate and, therefore, the spread of the vector to nonvaccinated contacts or to the general environment.

For reasons still not understood, it was demonstrated that a recombinant canarypox vector was a 100 times more efficient than a comparable fowlpox vector in inducing protective immunity and similar to a thymidine kinase-disrupted replication competent vaccinia virus vector (8).

Numerous examples have now been provided demonstrating the safety, immunogenicity, and protective efficacy of canarypox-based recombinants in both experimental animal models and target species. A prime example has used canarypox-based recombinants expressing the rabies virus glycoprotein G. Rabies virus infection and immunization are issues for both veterinary and human medicine. A great deal of information is available in rabies virus immunization, experimental animals and target species are readily available for study, and the parameters of successful immunization are understood. The safety and immunogenicity of a canarypox-based rabies glycoprotein recombinant was demonstrated in a number of nonavian species (9). Protection of vaccinated experimental animals or target species cats and dogs was demonstrated.

To appreciate the duration of immunity that could be engendered by vaccination with a canarypox-based recombinant, naive beagles were vaccinated by a single subcutaneous dose of the vaccine followed by rabies challenge with rabies virus. All vaccinated dogs seroconverted with maximal titers at 1 month. At various times after vaccination, a subset of dogs was challenged. At 6 and 12 months postvaccination, all dogs vaccinated with a single dose of the vaccine resisted challenge that was lethal to all the control animals. At 24 months after vaccination, 11 of 12 vaccinated dogs survived challenge with similar protection observed at 36 months postvaccination (10). These studies demonstrated that a single vaccination was immunogenic and that a protective immune response was primed such that recall as long as 3 years later was protective against a rabies virus challenge in the target species.

Successful vaccination in the presence of rabies-specific maternal antibodies was demonstrated in the following experiment using beagles. A worst scenario situation was established wherein pregnant bitches with immunity to rabies were revaccinated 2 weeks before whelping to maximize the antirabies antibody titers transferred from the bitch to the offspring. At 2 weeks after birth, the pups were vaccinated with a single dose of a canarypox-based rabies vaccine recombinant. Serological responses were followed to monitor either the decay of maternal antibodies in the nonvaccinated control pups or the effect on antibody titers on the pups vaccinated in the presence of maternal antibodies. At 3 months, immunity was challenged by inoculation of live rabies virus in the temporal muscle. The maternal antibody titer in the unvaccinated pups decayed with the expected kinetics. Pups vaccinated with the recombinant virus showed a slight increase in rabies virus neutralizing titer at 2 weeks postvaccination that fell to undetectable levels at the time of challenge. In a vaccine dose-dependent fashion, pups immunized in the presence of maternal immunity survived the rabies virus challenge that was lethal to all the nonvaccinated pups (10). This study demonstrated that young animals could be successfully vaccinated in the presence of maternal immunity.

The concept of using a nonreplicating avipox virus vector, a canarypox-based rabies recombinant, has been evaluated for safety and immunogenicity in human clinical studies (11, 12). Rabies naive healthy adult volunteers were inoculated with increasing doses of the recombinant in a schedule including a boost at 1 and 6 months. For comparison, the standard inactivated human diploid cell rabies vaccine was used. All inoculations with the recombinant canarypox vaccine were well-tolerated with only mild and short-lived reactions at the inoculation site reported. In these two clinical trials, induction of antirabies immune responses were demonstrated, and it was demonstrated that canarypox recombinants could be used either by themselves or in a protocol wherein the priming vaccination with the vector could be followed by a booster with the inactivated rabies vaccine.

Although the immune responses to the experimental canarypox recombinant were comparable but not demonstrated to be superior to those obtained with the standard inactivated rabies vaccine, it perhaps is not surprising given the relative low doses of the recombinant vaccine used in these studies and the comparison with an optimized and highly immunogenic licensed vaccine.

Other examples demonstrating the utility of canarypox virus-based vectors for veterinary species have been provided. Canarypox virus recombinants expressing the measles virus fusion and hemagglutinin glycoproteins have been used to vaccinate dogs. Comparison of these recombinants with vaccinia virus vectors expressing the same genes were shown to provide similar levels of immune response and protection against a challenge with the related Morbilli virus, canine distemper (13).

Construction of specific canine distemper virus recombinants expressing the fusion and hemagglutinin have been evaluated in the highly susceptible ferret model and dog host and were demonstrated to provide protection against challenge (unpublished data).

Canarypox-based recombinants expressing the hemagglutinin from equine influenza virus were shown to be immunogenic when inoculated in horses and provided protection against a naturally occurring equine influenza virus infection (14).

Two canarypox virus-based recombinants were constructed, each expressing the entire *gag* gene and either the intact subgroup A *envelope* of feline leukemia virus (FeLV) or a modified version of the *envelope* from which the putative immunosuppressive region was deleted (15). These recombinants were evaluated for protective efficacy in kittens of 8–9 weeks of age. Two inoculations of the recombinants at 5 and 2 weeks before challenge failed to induce measurable FeLV neutralizing antibodies. Nevertheless, 50% of the cats receiving the mutated *envelope* recombinant and 100% of the cats receiving the intact *envelope* recombinant were protected against an oronasal challenge with the FeLV-A/Glasgow-1 isolate. Protection was assessed by evaluating p27 antigenimea, detecting FeLV antigen in blood smears, and the attempted recovery of infectious FeLV. This was the first description of

a successful immunization against a retrovirus provided by pox virus-based recombinants.

The above observations provided an impetus to further investigate the potential of canarypox-based vectors for immunization against other retrovirus with particular attention on the lentiviruses with focus on HIV, the causative infectious agent of AIDS. The entire *envelope* protein of the human T-cell leukemia/lymphoma virus type I was expressed in a canarypox virus vector. Two inoculations of the recombinant vaccine candidate were administered to rabbits. Five months after the last inoculation, the animals were exposed to a human T-cell leukemia type-I cell associated challenge from a primary culture of the *bou* isolate. The animals were protected. The protected animals were again challenged 5 months after the initial challenge exposure with 5 ml of blood from an infected rabbit. Immunity failed this relatively large challenge exposure. Of interest in these studies (16) was the observation that if a subunit *envelope* booster was administered in alum after the priming vaccination with the canarypox recombinant protection was not obtained. Interpretation of this observation can lead to interesting speculation.

Other interesting observations using canarypox-based recombinants expressing antigens from either HIV-I or II, as well as simian immunodeficiency virus, have been reported. In laboratory rodents, induction of both humoral immunity as well as cytotoxic T lymphocyte (CTL) can readily be demonstrated (17).

Recombinants expressing HIV-II *gag*, *pol*, or *envelope* genes have been evaluated in macaques in several studies with some level of protection described (18, 19). Significant and raising concerns for those involved in vaccine development correlates of protective immunity are not revealed in these studies. Multiple immunization allowing for the maturation of the immune response is suggested by some studies (20). An intriguing observation was the cross protection against HIV-II challenge in monkeys vaccinated with HIV-1 recombinant pox viruses (21). A likely interpretation of this data is the induction of and protection by cross-reactive CTL. However, the basis of this cross protection is currently unknown.

A series of recombinant canarypox virus-based recombinants expressing an increasing complexity of HIV-I strain MN antigens have been constructed and evaluated in human clinical trials for both safety and immunogenicity. The earliest of these studies in HIV seronegative healthy adult volunteers have been reported (22). A vaccine regimen providing the best results to date involve one or two doses of the recombinant canarypox virus vector followed by one or two doses of an adjuvanted recombinant *envelope* subunit. The induction of binding, HIV neutralizing, and both CD4 and CD8 CTL have been reported (22–24).

More recent data using the more complex recombinants and higher doses of vaccine in a vector prime/subunit antigen boost protocol have demonstrated better levels of neutralizing antibody induction and a more complex reactivity of CTL to multiple HIV antigens. Further comparison of separate phase I trial data a prime/boost protocol using the canarypox vector fares favorably when compared with a prime boost protocol using a replication competent vaccine vector as a primer (unpublished data). In this light, the failure of the canarypox vector to replicate in the mammalian host provides advantage over the replication competent vaccinia virus vector. The general safety profile of the HIV-I canarypox recombinants in human volunteers is similar to that observed with the canarypox recombinants expressing the rabies virus glycoprotein discussed above.

Attenuated Vaccinia-Based Vector: NYVAC

The global smallpox eradication program was made possible by several biological features of the pathogen and the vaccine. The pathogen had only a single host for infection and propagation—man. There were no animal reservoirs from which the pathogen could recrudesce. Defined outbreaks of the infection could be circumscribed and contained by vaccination. Vaccinia, the vaccine, could be produced efficiently and at low cost in regional centers. The ability to retain potency of the vaccine as a freeze-dried preparation allowed storage and transport to remote regions of the globe. The successful smallpox eradication program, however, was not without vaccine-associated risk. Vaccine reactogenicity with some severe or lethal outcomes was associated with the vaccine in general and specifically higher rates of adverse events were evidenced in certain populations or with certain vaccine strains or preparations. Early attempts to manufacture the vaccine under more defined and regulated laboratory conditions were abandoned with the success of the eradication effort. The known reactogenicity of the vaccinia vaccine was therefore a concern to be addressed when the virus was proposed as a vector for new engineered vaccines. This concern has been addressed in several ways such as the provision of naturally host-restricted vectors described above or by the targeted attenuation of existing vaccine strains. This approach is demonstrated by the engineering of the NYVAC strain of vaccinia virus. The Copenhagen strain of vaccinia was chosen as a vaccine substrate. The entire DNA sequence of the genome was established (25). With this information and the extant knowledge of virulence-related and other genetic functions related to host range replication competency unwanted genetic information was precisely deleted from the vaccinia virus genome. The resultant vector, NYVAC, was highly attenuated as demonstrated in a series of studies in animal surrogates (26). Intracranial inoculation of newborn or young adult mice demonstrated a very favorable dose range compared with either the parent or other vaccine strains, and significantly no disseminated viral infection was observed in immunocompromised hosts. In numerous tissue culture cells of human origin, the vector was shown to be highly debilitated for replication consistent with the deletion of host range genes. The modified NYVAC vector, while highly attenuated, retained the ability to induce protective immune responses to foreign antigens in a fashion similar to the thymidine kinase mutant of the parent strain.

A number of examples using the NYVAC vector as a recombinant vaccine delivery system have been provided in animal model systems and in target species including humans. A series of NYVAC recombinants were generated to express glycoproteins from Pseudorabies virus (PRV) and the immunity afforded by these recombinants was evaluated in the target species of PRV infection, the pig. PRV neutralizing antibodies were induced following two intramuscular inoculations 28 days apart. The NYVAC recombinant expressing the PRV glycoprotein gp50 induced levels of PRV neutralizing antibodies and afforded protection against a virulent oronasal PRV challenge that was comparable to vaccination with inactivated PRV vaccine (27). The advantage of a recombinant vaccine is that one is allowed to discriminate between a naturally infected versus vaccinated animal since the recombinant vaccine displays a defined subset of the antigens of the pathogens. This discrimination allows the agricultural industry to properly track infections and cull infected herds.

A NYVAC-based recombinant expressing two hemagglutinin glycoproteins of the A1 and A2 equine influenza serotypes induced hemagglutinin inhibiting antibodies when inoculated into horses and afforded significant protection when the vaccinated horses became exposed to a natural equine influenza virus infection (14).

The polyprotein of Japanese encephalitis virus (JEV) encoding prM/M, E, and NS1 was expressed in NYVAC recombinants and the vector used to vaccinate swine, a major natural host of JEV infection and a reservoir for mosquito transmis-

sion of the virus to man. Hemagglutinin-inhibiting and JEV-neutralizing antibodies were induced on vaccination. The nonvaccinated challenged animals succumbed to JEV infection, whereas the vaccinated group had levels of JEV challenge viremia insufficient to be transmitted by mosquitoes (28) Both a NYVAC- and a canarypox-based Japanese encephalitis recombinant are currently being evaluated in human clinical trials.

A NYVAC vector has been engineered to express the rabies glycoprotein gene. In mice, cats, and dogs, the recombinant was shown to be safe and to provide protection against a lethal rabies virus challenge. The recombinant is now being evaluated in phase I human clinical trials for safety and immunogenicity.

Pox virus vectors have been used to determine the immunogenic potential of antigens from *Plasmodium* spp. in an effort to understand the design of an effective vaccine against malarial infections. In this regard a NYVAC vector reconstituted with the K1L host range gene was constructed to express intact or mutated forms of the circumsporozoite protein of *Plasmodium berghei*. Vaccination of the target host, the mouse, induced both binding antibody and CTL. Vaccinated and control mice were challenged either by the intravenous injection of sporozoites or by allowing infected mosquitoes to feed on the subjects. Protection was scored as the absence of blood stage parasetemia as determined by microscopic analysis of blood films from individual mice from 5–15 days after challenge. In a number of challenge experiments, ≈80% protection was obtained. This is to be compared with the consistent 100% level of protection obtained by vaccination with irradiated sporozoites. Protection in the recombinant virus-immunized mice apparently did not correlate with antibodies but a good correlation was established between CTL and protection. *In vivo* antibody depletion of $CD8^+$ T cells before challenge abrogated protection (29).

With this data as an inducement, a complex NYVAC-based recombinant was constructed to express multiple antigens from *P. falciparum*. To address the multiple stages of the parasite life cycle, multiple antigens from the various stages were used. Thus, a recombinant expressing seven parasite antigens was provided. This recombinant was evaluated in rodents and in monkeys where safety and immunogenicity were established (30). This recombinant is now being evaluated in clinical trials where the vaccinated subjects are exposed to the bites of infected mosquitoes. Appearance of parasites in the blood of the infected volunteers will terminate the challenge followed by administration of antimalarial drugs to thwart further replication of the parasite. Since ethical and medical considerations require treatment on appearance of blood-stage parasites, only the antisporozoite and liver-stage immunity engendered by the vaccine can be evaluated. Full evaluation of blood-stage and transmission-blocking immunity cannot be evaluated in this limited clinical setting.

To date, all the above-mentioned abstracted data provided from human clinical trials using NYVAC-based vectors have described a good safety profile and the induction of some level of immunity to the expressed heterologous antigens.

Other Applications of Pox Virus-Based Vectors

The use of pox virus-based vectors as recombinant vaccines for heterologous bacterial, viral, or parasitic pathogens was the first practical application of this technology deriving from the fact that vaccinia virus was an established vaccine. However, the pox virus vectors can be looked at as general delivery systems for genes for other applications. For example, these vectors can be used *in vitro* to stimulate and expand CTL reactivities from the peripheral blood of chronically infected or tumor-bearing individuals (31). The antigen-specific stimulation and expansion of such cultures might provide some therapeutic benefit when reintroduced to the donor patient.

For cancer immunotherapy, numerous pox virus-based recombinants expressing tumor-associated antigens or biological response modifiers have been described (32). Of particular note, recombinants expressing the carcinoembryonic antigen were shown to elicit both antibody and cellular immune responses in mice and monkeys and to protect mice from tumor cell challenge (33, 34). Whether vaccinia or canarypox-based recombinants expressing the carcinoembryonic antigen will have any therapeutic benefit is currently being investigated in the clinic in patients with colorectal carcinomas.

A recent publication (4) reported the protection of mice vaccinated with a p53 expressing recombinant against challenge with an isogenic and highly tumorigenic mouse fibroblast tumor cell line expressing high levels of a mutant human p53 but lacking endogenous murine p53. Expression of the mutant form of p53 in the recombinant virus was not essential since the wild-type p53 afforded similar efficacy. This may be an important observation since p53 is an attractive target for cancer immunotherapy. Mutations of p53 represent the most common genetic changes demonstrated in human tumors.

Discussion

The excitement of the 1982 proposal to use pox virus-based vectors as heterologous vaccines and the ensuing years of extensive pursuit of this idea have provided numerous working examples in laboratory animal model systems as well as in target species. In the veterinary field, products have now been licensed for commercialization and a significant number of clinical studies have been and continue to be pursued for both infectious diseases, *ex vivo* therapies, and cancer immunotherapy. The immediate future looks to be as exciting as the recent past.

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